

## 红芽芋茎尖的包埋玻璃化法超低温保存\*

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**摘要:** 对红芽芋 (*Colocasia esculenta* var. *cormosus* 'Hongyayu') 茎尖的包埋玻璃化法超低温保存技术进行了研究。茎尖从培养 8 周的试管苗上切下并包埋成海藻酸钙凝胶珠, 并在  $MS+3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA+ $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub>+ $0.3\text{ mol}\cdot\text{L}^{-1}$  蔗糖的液体培养基中预培养 24 h, 随后用  $2\text{ mol}\cdot\text{L}^{-1}$  甘油+ $0.4\text{ mol}\cdot\text{L}^{-1}$  蔗糖的混合物在 25 °C 下装载 30 min, 并用 PVS2 在 25 °C 脱水 20 min 后将包埋的茎尖直接投入液氮保存。保存 1 d 后取出材料在 40 °C 水浴快速复温 3 min 后, 吸去冷冻管中 PVS2, 并用  $MS+3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA+ $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub>+ $1.2\text{ mol}\cdot\text{L}^{-1}$  蔗糖的液体培养基在 25 °C 洗涤 3 次, 每次 10 min。最后将茎尖接种于  $MS+3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA+ $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA+ $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub> 的固体培养基上, 暗培养 3 d 后转入正常的光周期中培养。红芽芋茎尖冻后成活率约为 80%, 其再生植株没有发生形态学的变化。这种包埋玻璃化法程序有望成为红芽芋茎尖超低温保存的常规方法。

**关键词:** 超低温保存; 包埋玻璃化法; 红芽芋; 茎尖

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## Cryopreservation of *in vitro*-Grown Shoot Tips of Red Bud Taro by Encapsulation-Vitrification

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**Abstract:** A simple procedure for cryopreservation of *in vitro*-grown shoot tips of red bud taro (*Colocasia esculenta* L. Schott var. *cormosus* 'Hongyayu') by encapsulation-vitrification is investigated. Shoot tips were excised from 8-week-old stock shoots and encapsulated into alginate-gel beads. Encapsulated shoot tips were precultured in liquid MS medium supplemented with  $3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA,  $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA,  $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub> and  $0.3\text{ mol}\cdot\text{L}^{-1}$  sucrose for 24 h, then they were loaded with a mixture of  $2\text{ mol}\cdot\text{L}^{-1}$  glycerol plus  $0.4\text{ mol}\cdot\text{L}^{-1}$  sucrose for 30 min at 25 °C. After dehydration with PVS2 at 25 °C for 20 min, the encapsulated and dehydrated shoot tips were plunged directly into liquid nitrogen. After rapidly rewarming in a 40 °C water bath for 3 min, PVS2 was drained from the cryotubes and replaced third with liquid MS medium supplemented with  $3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA,  $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA,  $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub> and  $1.2\text{ mol}\cdot\text{L}^{-1}$  sucrose and each kept for 10 min at 25 °C and then post-cultured on solidified MS medium supplemented with  $3.5\text{ mg}\cdot\text{L}^{-1}$  6-BA,  $0.5\text{ mg}\cdot\text{L}^{-1}$  IBA and  $0.1\text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub> in the dark for 3 days and then transferred to the light conditions. The average survival rate amounted to about 80%. Plantlets regenerated from cryopreserved shoot tips were morphologically uniform. This encapsulation vitrification procedure promises to become a routine method for the cryopreservation of shoot tips of Chinese genuine red bud taro.

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**Key words:** Cryopreservation; Encapsulation-vitrification; Red bud taro (*Colocasia esculenta* var. *cormosus* ‘Hongyayu’); Shoot-tips

*Colocasia esculenta* L. Schott var. *cormosus* ‘Hongyayu’ (Araceae), known as red bud taro in China, is one of the most economically important herbaceous, vegetatively propagated tuber crop (Li, 2012). All parts of the plant including corm, stalk, leaves and flowers are edible and contain abundant starch, protein, vitamin, polysaccharides and various trace elements and have been used as an immunostimulant (Jiang *et al.*, 2012). In recent years, it has also become a popular tonic and health food, therefore the demand for red bud taro has increased dramatically. In China, *C. esculenta* L. Schott var. *cormosus* ‘Hongyayu’ is a genuine taro belong to Yanshan County, Shangrao City, Jiangxi Province and mainly cultivated in the southeast of China (Xiao, 2006), Yanshan County is the only county red bud taro green food raw material standard production base, whose red bud taro was awarded national pollution-free green food certificate. At present, the planting area of red bud taro in Yanshan County is  $68 \times 10^4$  ha, with an annual output of 150 000 tons. Yanshan County has become the biggest red bud taro base in East China area.

As with any clonally propagated crop, germplasm resources of red bud taro are mainly conserved vegetatively in field gene banks or greenhouse. Jiangtian agricultural science and technology limited company in Yanshan County of Jiangxi Province has a breeding project of red bud taro (Li *et al.*, 2009). In this case, however, valuable materials are often exposed to pests, diseases, and soil or climatic stresses. In addition, the routine maintenance of plants in field gene banks is highly expensive due to the large size of these plants. There is a growing need for cryopreservation of the germplasm collections of red bud taro.

For many crops, cryopreservation is currently being applied to overcome the limitations encountered by traditional germplasm conservation strategies

in field (Engelmann-Sylvestre and Engelmann, 2015). Cryopreservation, i. e. storage at the temperature of liquid nitrogen, is an increasingly important aspect of plant biotechnology, since, in the future it will be necessary to establish safe repositories for patented cultures of commercial interest (Liu *et al.*, 2015; Vieira *et al.*, 2015). Further, this method enables us to preserve tissue cultures with valuable morphogenetic or biochemical traits (Panta *et al.*, 2015).

In recent years, cryopreservation procedures such as vitrification, encapsulation-dehydration, droplet-vitrification and encapsulation-vitrification have been developed (Sakai and Engelmann, 2007; Kaczmarczyk *et al.*, 2011; Tahtamouni *et al.*, 2015; Gantait *et al.*, 2015). Encapsulation-vitrification, generally including preculture, loading, dehydration, cooling, rewarming and post-culture, is a simplified cryostorage procedure because it does not require the use of expensive cooling devices (Shin *et al.*, 2014) and the encapsulated materials are much easier to manipulate and permit greater flexibility in handling large amounts of material (Sharaf *et al.*, 2012; Gogoi *et al.*, 2012). Recently the cryopreservation by encapsulation-vitrification has been applied to a wide range of plant species such as apple (Paul, 2000), mint (Hirai and Sakai, 1999a), potato (Hirai and Sakai, 1999b), strawberry (Hirai *et al.*, 1998), ‘Troyer’ citrange (Wang *et al.*, 2002), cassava (Charoensub *et al.*, 2004), carnation (Halmagyi and Deliu, 2007).

Cryopreservation has also been applied to taro genetic resources. Shimonishi *et al.* (1993) report for the first time the cryopreservation of taro embryogenic callus by slow cooling. However, shoot tips are better materials compared with cell and callus cultures because they result in true-to-type progeny in higher frequency than cultured cells and callus and have no genetic changes during the growth phase before and after storage (Sharaf *et al.*, 2012). In re-

cent years, the use of taro shoot-tips for germplasm cryopreservation has been achieved by vitrification (Takagi *et al.*, 1994, 1997, 1998; Sant *et al.*, 2006) and by droplet vitrification (Sant *et al.*, 2008).

After cryopreservation by vitrification and the average rate of taro shoot recovery amounted to around 80% (Takagi *et al.*, 1997). While the best mean recoveries for three cultivars of tropical taro (*Colocasia esculenta* var. *sculenta* (L.) Schott) were only 21%, 29% and 30% for E399, CPUK and TNS, respectively and cryopreservation by vitrification was evaluated with five other taro cultivars with no success (Sant *et al.*, 2006). The application of the droplet vitrification cryopreservation technique to taro accessions from a range of Asia Pacific countries significantly improved the mean post-thaw survival rates to 73%–100% from 21%–30% obtained with the cryo-vial vitrification protocol (Sant *et al.*, 2008). Therefore, for different varieties of taro, different cryopreservation procedures need further improvement. However, to our knowledge, there are no reports on the cryopreservation of red bud taro shoot tips by encapsulation-vitrification. The objective of the present study was, therefore, to develop a simple effective method for the cryopreservation of shoot tips of *C. esculenta* L. Schott var. *cormosus* ‘Hongyayu’ by encapsulation-vitrification.

## 1 Materials and methods

### 1.1 Plant materials and *in vitro* culture

Field-grown cormels of *C. esculenta* L. Schott var. *cormosus* ‘Hongyayu’ were obtained from Jiangtian agricultural science and technology limited company, Yanshan County, Jiangxi Province, China. Firstly, the cormels were immersed in 70% ethanol for 20 s followed by 0.1%  $\text{HgCl}_2$  for 12 min and washed three times with sterile distilled water respectively. *In vitro* stock plantlets of *C. esculenta* L. Schott var. *cormosus* ‘Hongyayu’ were obtained by culturing 0.3–0.6 mm-long shoot tips, isolated from sterilized cormels (Takagi *et al.*, 1997) and propa-

gated on solidified MS (Murashige and Skoog, 1962) basal medium supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$   $\text{GA}_3$ ,  $20 \text{ g} \cdot \text{L}^{-1}$  sucrose and  $6.5 \text{ g} \cdot \text{L}^{-1}$  agar at pH 5.8 (Li, 2012). Shoots from the *in vitro*-grown stock plants were proliferated on solidified MS basal medium containing  $3 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  NAA,  $20 \text{ g} \cdot \text{L}^{-1}$  sucrose and  $6.5 \text{ g} \cdot \text{L}^{-1}$  agar at pH 5.8 (Li, 2012; Takagi *et al.*, 1997). The shoots constituted the stock materials used for subsequent investigations and were subcultured every 8 weeks. All of the cultures were maintained at a constant temperature of  $25^\circ\text{C}$  and exposed to a 16/8-h (light/dark) photoperiod with a light intensity of  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  provided by cool-white fluorescent tubes. Immediately prior to use, shoot tips, 0.8–1.0 mm long containing 1 leaf primordia, were excised from axillary buds of 8-week-old stock shoots (Takagi *et al.*, 1997; Sant *et al.*, 2006, 2008).

### 1.2 Cryopreservation

Excised shoot tips were suspended in calcium-free MS inorganic medium supplemented with 2% Na-alginate and  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose. The mixture including shoot tips was dispensed with a sterile pipette into  $0.1 \text{ mol} \cdot \text{L}^{-1}$   $\text{CaCl}_2$  plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose solution for 30 min at  $25^\circ\text{C}$  to form Ca-alginate beads (4 mm in diameter). Each bead contained one shoot tip. These beads were surface-dried by plating them on a sterilized filter paper and then precultured.

Shoot tips were precultured after being excised from 8-week-old stock shoots and encapsulated into alginate-gel beads. The following parameters of the preculture protocol were studied:

(1) Effect of sucrose concentration in preculture medium: Encapsulated shoot tips were precultured in a liquid MS medium containing various concentrations of sucrose (0, 0.15, 0.3, 0.45, 0.6  $\text{mol} \cdot \text{L}^{-1}$ ) supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$   $\text{GA}_3$  for 24 h.

(2) Effect of sucrose preculture duration: Encapsulated shoot tips were precultured in a liquid MS

medium containing  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> for 0, 12, 24, 48, 60 h respectively.

Precultured shoot tips were loaded with a mixture of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose at 25 °C for 0, 10, 20, 30, 40, 50, 60 min respectively. Cryoprotected shoot tips were transferred to a 2 mL plastic cryotube (Containing 5 beads) and 1 mL-PVS2 was then added.

The following parameters of the dehydration protocol were studied:

(3) Effect of dehydration duration at 25 °C: Encapsulated, precultured and loaded shoot tips were dehydrated with PVS2 at 25 °C for different intervals (0, 10, 20, 30, 40, 50, 60 min).

(4) Effect of dehydration duration at 0 °C: Encapsulated, precultured and loaded shoot tips were dehydrated with PVS2 at 0 °C for different lengths of time (0, 10, 20, 30, 40, 50, 60 min).

After dehydration with PVS2, the encapsulated and dehydrated shoot tips were plunged directly into liquid nitrogen (LN) for 1 d.

After rapidly rewarming in a water bath at 25 °C, 40 °C and 45 °C for 3 min respectively, PVS2 was drained from the cryotubes and washed third with liquid MS medium containing various concentrations of sucrose (0, 0.4, 0.8, 1.2, 1.6  $\text{mol} \cdot \text{L}^{-1}$ ) supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> (pH 5.8) and each kept for 10 min at 25 °C and then post-cultured on solidified MS medium supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA and  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub>. The following parameters of the post-culture protocol were studied:

(5) Effect of 3-day dark culture prior to 16 h photoperiod: Encapsulated, precultured, loaded, dehydrated, cryopreserved, thawed, loaded shoot tips were post-cultured in the dark for 3 days and then transferred to the light conditions (under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ).

(6) Effect of 16 h photoperiod without 3 day dark culture: Encapsulated, precultured, loaded, dehydrated, cryopreserved, thawed, unloaded shoot

tips were directly post-cultured under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ .

Surviving shoot tips were transferred to solidified MS medium containing  $3 \text{ mg} \cdot \text{L}^{-1}$  6-BA and  $0.5 \text{ mg} \cdot \text{L}^{-1}$  NAA under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for shoot growth and after one month the shoots transferred to solidified hormone-free MS rooting medium under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . The survival rate was estimated as the percentage of the total number of shoot tips that developed a plantlet after 4 months culture.

The recovery and plant regeneration of cryopreserved shoot tips was studied by the following two groups:

(7) Treatment group: Shoot tips were excised from 8-week-old stock shoots and encapsulated into alginate-gel beads. Encapsulated shoot tips were pre-cultured in a liquid MS medium containing  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> for 24 h and then loaded with a mixture of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose for 30 min at 25 °C. Cryoprotected shoot tips were transferred to a 2 mL plastic cryotube and PVS2 was then added. After dehydration with PVS2 at 25 °C for 20 min, the encapsulated and dehydrated shoot tips were plunged directly into liquid nitrogen (LN) for 1 d. After rapidly rewarming in a 40 °C water bath for 3 min, PVS2 was drained from the cryotubes and replaced third with liquid MS medium supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> and  $1.2 \text{ mol} \cdot \text{L}^{-1}$  sucrose (pH 5.8) and each kept for 10 min at 25 °C and then post-cultured on solidified MS medium supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA and  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> in the dark for 3 days and then transferred to the light conditions (under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Surviving shoot tips were transferred to solidified MS medium containing  $3 \text{ mg} \cdot \text{L}^{-1}$  6-BA and  $0.5 \text{ mg} \cdot \text{L}^{-1}$  NAA under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for growth and after one month the shoots transferred to solidified hormone-free MS rooting medium under 16 h

photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ .

(8) Control group: Shoot tips were excised from 8-week-old stock shoots and cultured on solidified MS medium supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA and  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> in the dark for 3 days and then transferred to the light conditions (under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Surviving shoot tips were transferred to solidified MS medium containing  $3 \text{ mg} \cdot \text{L}^{-1}$  6-BA and  $0.5 \text{ mg} \cdot \text{L}^{-1}$  NAA under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for growth and after one month the shoots transferred to solidified hormone-free MS rooting medium under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ .

After 4 months culture, various phenotypic characteristics (height of plantlet, length of root, number of root, and number of leaf) of the regenerated plantlets of the treatment group and the control group were estimated.

### 1.3 Statistical analysis

Each cryopreservation experiment included at least 15 shoot tips. Each cryopreservation experiment was repeated three times. All data were subjected to analysis of variance (one way ANOVA) and significance ( $P < 0.05$ ) was determined with Duncan's multiple range test. Statistical tests were performed by the help of SPSS statistical package version 19.0.

## 2 Results

### 2.1 Effect of preculture duration and sucrose concentration in preculture medium on regeneration

The results (Fig. 1) demonstrate that high sucrose concentrations ( $0.3 - 0.45 \text{ mol} \cdot \text{L}^{-1}$ ) during preculture improve survival rate of shoot tips after cryopreservation. Survival rates above 80% were only noted with sucrose concentrations of 0.3 to  $0.45 \text{ mol} \cdot \text{L}^{-1}$ . The survival rate of 82.5% obtained with  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose differed significantly from that noted with other sucrose concentrations, and  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose can thus be considered optimal for re-growth. Survival decreased to 37.6% in the presence of  $0.6 \text{ mol} \cdot \text{L}^{-1}$  sucrose and to 21.5% with  $0 \text{ mol} \cdot \text{L}^{-1}$  sucrose and to 42.1% with  $0.15 \text{ mol} \cdot \text{L}^{-1}$  sucrose.

Thus, preculture using liquid MS medium containing  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> for 24 h was an important step for the successful cryopreservation of red bud taro by encapsulation-vitrification. As shown in Fig. 2, the survival rate increased with increasing preculture time, highest survival rate was achieved (84.2%) when preculturing for 24 h, and then rapidly declined when preculturing for 48 h or 60 h. At the same time, The benefit of preculture shoot tips before their loading was also tested using liquid MS medium containing various concentrations of sucrose supplemented with  $3.5 \text{ mg} \cdot \text{L}^{-1}$  6-BA,  $0.5 \text{ mg} \cdot \text{L}^{-1}$  IBA,  $0.1 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> for 24 h.

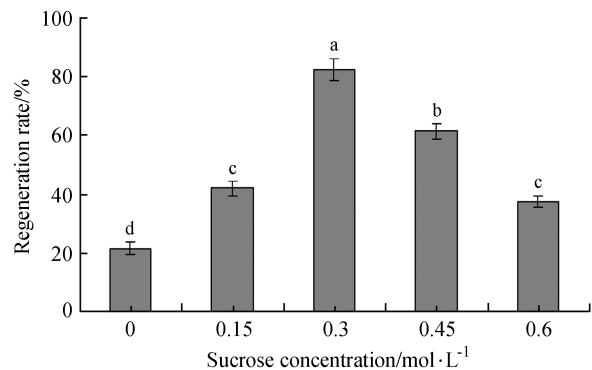


Fig. 1 Effect of sucrose concentration in preculture medium on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications.

Values with different letters are significantly different using Duncan's Multiple Range Test ( $P < 0.05$ )

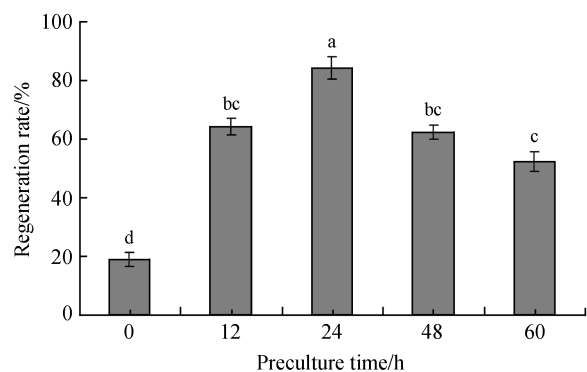


Fig. 2 Effect of preculture time on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different

letters are significantly different using Duncan's

Multiple Range Test ( $P < 0.05$ )

## 2.2 Effect of duration of loading treatment on regeneration

To determine the appropriate loading time for red bud taro shoot tips, different periods of time in a mixture of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol and  $1.6 \text{ mol} \cdot \text{L}^{-1}$  sucrose at  $25^\circ\text{C}$  before dehydration with PVS2 were examined. As shown in Fig. 3, loading was very effective in improving the survival rate of encapsulated vitrified shoot tips cooled to  $-196^\circ\text{C}$  in comparison with the unloaded shoot tips. Survival rate was 0 for non-loaded shoot tips. Precultured shoot tips which were loaded for 30 min produced the highest percentage of shoot regeneration (79.8%). Increasing the loading period from 40 to 60 min, the shoot tip survival rate decreased instead. Based on this result,

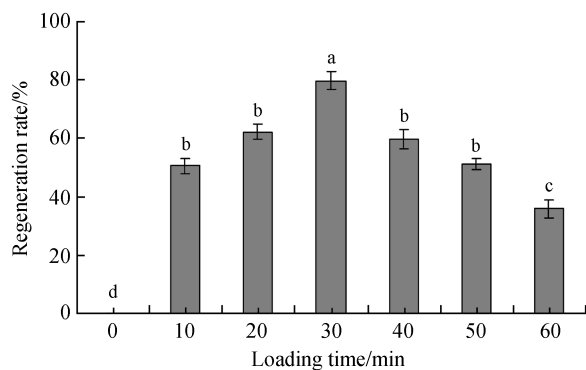


Fig. 3 Effect of loading time on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different letters are significantly different using Duncan's Multiple Range Test ( $P < 0.05$ )

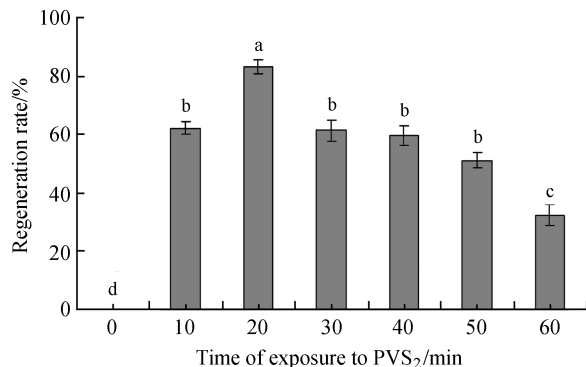


Fig. 4 Effect of exposure to PVS<sub>2</sub> at  $25^\circ\text{C}$  on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different letters are significantly different using Duncan's Multiple Range Test ( $P < 0.05$ )

a 30 minute loading time of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose was adopted as the loading time for red bud taro shoot tips in subsequent experiments.

## 2.3 Effect of duration and temperature of exposure to PVS2 on regeneration

To optimize the duration and temperature of exposure to PVS2 controlling the regeneration of shoot tips during encapsulation-vitrification, we dehydrated encapsulated, precultured and loaded shoot tips with PVS2 solution for various lengths of time at  $0^\circ\text{C}$  and  $25^\circ\text{C}$  before immersion in LN. As shown in Fig. 4 and Fig. 5, exposure to PVS2 for 50 min at  $0^\circ\text{C}$  or 20 min at  $25^\circ\text{C}$  gave high survival rate for the shoot tips tested, but the highest rate of shoot regeneration (83.2%) was obtained by exposure to PVS2 for 20 min at  $25^\circ\text{C}$ . Based on statistical analysis, this value is significantly different from that (71.1%) of shoot tips treated for 50 min at  $0^\circ\text{C}$  ( $P < 0.05$ ). However, exposure to PVS2 for 30 to 60 min at  $25^\circ\text{C}$  or for up to 60 min at  $0^\circ\text{C}$  decreased shoot survival rate. Thus, in all experiments, shoot tips were previously dehydrated with PVS2 for 20 min at  $25^\circ\text{C}$  prior to a plunge into LN.

## 2.4 Effect of cryopreserved storage duration on regeneration

In order to examine the regeneration of the shoot tips for long term storage in LN, the shoot tips of red bud taro were cryopreserved in LN for 1 to 150 days

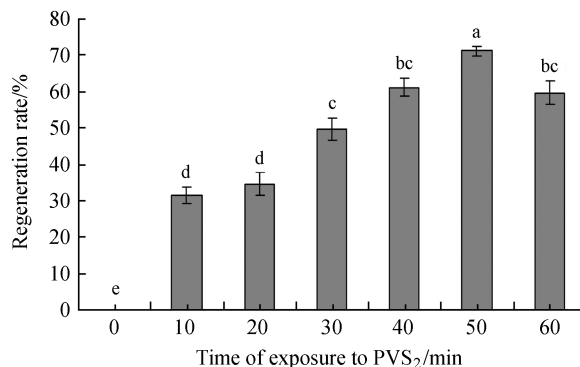


Fig. 5 Effect of exposure to PVS<sub>2</sub> at  $0^\circ\text{C}$  on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different letters are significantly different using Duncan's Multiple Range Test ( $P < 0.05$ )

by encapsulation-vitrification. Long term storage in LN did slightly vary the regeneration percentage of shoot tips surviving LN exposure (Fig. 6), statistically, however, the difference is not significant.

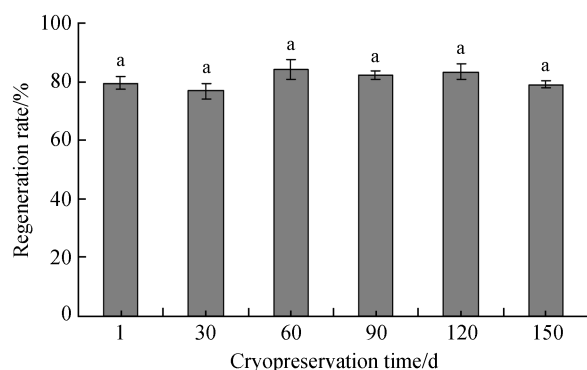


Fig. 6 Effect of cryopreservation time on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Mean comparisons of each survival rate were not significant ( $P>0.05$ )

## 2.5 Effect of rewarming on regeneration

To test the effect of rewarming on regeneration, encapsulated vitrified shoot tips in LN were thawed rapidly in a water bath at 25 °C, 40 °C and 45 °C for 3 min respectively. As shown in Fig. 7, rewarming rapidly in a water bath at 25 °C and 45 °C produced low levels of shoot survival rate (62.7% and 49.8% respectively). Rewarming at higher temperature (40 °C) had a much higher survival rate (80.9%) than that at lower temperature (25 °C and 45 °C).

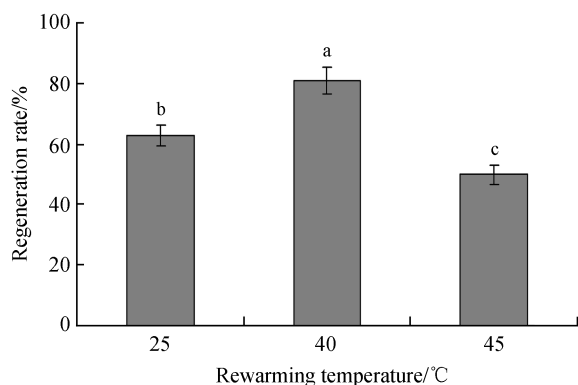


Fig. 7 Effect of rewarming temperature on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different letters are significantly different using Duncan's Multiple Range Test ( $P<0.05$ )

## 2.6 Effect of sucrose concentration in unloading medium on regeneration

To investigate the effect of sucrose concentration in unloading medium on regeneration, the cryopreserved shoot tips by encapsulation-vitrification was unloaded third with liquid MS medium containing various concentrations of sucrose supplemented with 3.5 mg · L<sup>-1</sup> 6-BA, 0.5 mg · L<sup>-1</sup> IBA, 0.1 mg · L<sup>-1</sup> GA<sub>3</sub> (pH 5.8) and each kept for 10 min at 25 °C. As shown in Fig. 8, regrowth after immersion in LN increased with the increasing of the concentration of sucrose in unloading medium and shoot tips unloaded with 1.2 mol · L<sup>-1</sup> sucrose showed the highest survival rate (85.4%).

## 2.7 Effect of post-culture on regeneration

The effect of post-culture on regeneration is shown in Fig. 9. The survival rate of cryopreserved shoot tips by encapsulation-vitrification varied considerably, depending on the light culture conditions after cooling. When shoot tips were post-cultured in the dark for 3 days and then transferred to the light conditions (under 16 h photoperiod at 36 μmol · m<sup>-2</sup> s<sup>-1</sup>), the survival rate was maximum (82.6%). However, when shoot tips were directly post-cultured under 16 h photoperiod the survival rate decreased to 69.8%.

## 2.8 Plant regeneration

Successfully encapsulated, vitrified and warmed

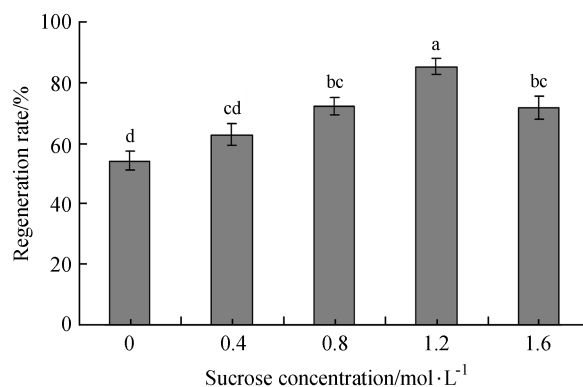


Fig. 8 Effect of sucrose concentration in unloading medium on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications.

Values with different letters are significantly different using Duncan's Multiple Range Test ( $P<0.05$ )

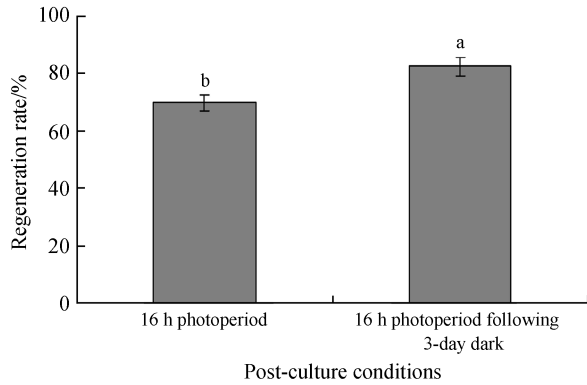


Fig. 9 Effect of post-culture on regeneration of cryopreserved red bud taro shoot tips by encapsulation-vitrification. Bars correspond to SE of means of three replications. Values with different letters are significantly different using Duncan's Multiple Range Test ( $P < 0.05$ )

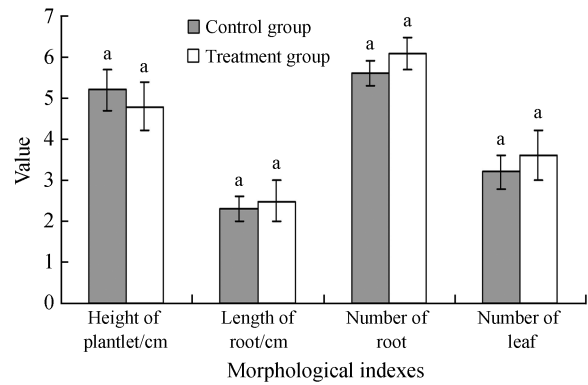


Fig. 10 Comparison of morphological indexes of treatment group (with cryopreservation) and control group (without cryopreservation). Bars correspond to SE of means of three replications. Mean comparisons of each of the morphological indexes between the two types of plantlets were not significant ( $P > 0.05$ )

shoot-tips remained green continuously after plating, resumed growth within a week and developed normal shoots directly within three weeks without intermediary callus formation. Surviving shoot tips were transferred to solidified MS medium containing  $3 \text{ mg} \cdot \text{L}^{-1}$  6-BA and  $0.5 \text{ mg} \cdot \text{L}^{-1}$  NAA under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for proliferation and after one month the shoots transferred to solidified hormone-free MS rooting medium under 16 h photoperiod at  $36 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and developed into plantlets within 2 months of culture. Various phenotypic characteristics (height of plantlet, length of root, number of root, and number of leaf) of the regenerated plantlets of the treatment group and the control group were estimated. As shown in Fig. 10 and Fig. 11, regenerated plants derived from cryopreserved shoot tips by encapsulation-vitrification were morphologically identical to controls and no morphological abnormalities were observed.

### 3 Discussion

The present results have demonstrated the feasibility for the cryopreservation of red bud taro (*C. esculenta* L. Schott var. *cormosus* 'Hongyayu') shoot tips by encapsulation-vitrification.

In the encapsulation-vitrification method, shoot tips are dehydrated by a highly concentrated vitrification solution (PVS2). However, the direct exposure



Fig. 11 Regeneration plantlets of treatment group (Left, with cryopreservation) and control group (Right, without cryopreservation)

of less tolerant shoot tips to PVS2 results in harmful effects due to osmotic stress and chemical toxicity (Sakai and Engelmann, 2007). The harmful effects due to dehydration can be alleviated or eliminated by adequate preculture with high concentration of sucrose (Sharaf *et al.*, 2012). Precultures of excised shoot tips with different concentrations of sucrose prior to loading treatment have been reported to be effective in improving post-LN survival rates of shoot-tips of some taro varieties. When shoot tips of three *C. esculenta* var. *esculenta* (L.) Schott cultivars (E399, CPUK and TNS) were precultured on MS with  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose in the dark for 16 h at  $25^\circ\text{C}$  prior to loading, the best recoveries were 21%, 29% and

30% respectively (Sant *et al.*, 2006). However, after 16 hours of preculture in  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose under a 16 h photoperiod at  $25^\circ\text{C}$  before cooling in LN by vitrification, the average recovery rate of *C. esculenta* var. *antiquorum* cv. ‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ shoot tips could reach 83.1% and 67% respectively (Takagi *et al.*, 1997). Therefore, preculture time and sucrose concentration in preculture medium are mainly depending on plant species and varieties. In the present study, culturing encapsulated red bud taro shoot tips in liquid MS medium supplemented with  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose for 24 h was effective in inducing dehydration and cooling tolerance. Preculturing for 12 h resulted in lower survival, which can be attributed to an insufficient acquisition of desiccation tolerance or cryoprotection (Florence *et al.*, 1993). Longer periods of preculture ( $>24 \text{ h}$ ) with  $0.3 \text{ mol} \cdot \text{L}^{-1}$  sucrose did not further improve the percentage of shoot formation, most likely due to tissue growth and changes in the physiological condition (Wang *et al.*, 2005a). Similar high sucrose concentration preculture also resulted in tolerance of PVS2 dehydration and subsequent cooling in droplet vitrified shoot tips of *C. esculenta* var. *esculenta* cultivars (E399, CUPUK and TNS, *et al.*) (Sant *et al.*, 2008). before cooling in LN by vitrification.

Following preculture, shoot tips must be loaded with a mixture of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose prior to exposure to PVS2 in order to prevent injury by excess osmotic stresses or chemical toxicity during dehydration (Hirai and Sakai, 1999a; Hong and Yin, 2012a). In previous studies, shoot tips of *C. esculenta* var. *esculenta* (L.) Schott cultivars (Sant *et al.*, 2006, 2008) and *C. esculenta* var. *antiquorum* cv. ‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ (Takagi *et al.*, 1997) have been successfully cryopreserved by loading for 20 min at  $25^\circ\text{C}$  before the exposure to PVS2. In the present study, a 30 min period of loading treatment with a mixture of  $2 \text{ mol} \cdot \text{L}^{-1}$  glycerol plus  $0.4 \text{ mol} \cdot \text{L}^{-1}$  sucrose at  $25^\circ\text{C}$  was also essential to produce a high survival rate in

cryopreservation by encapsulation-vitrification of red bud taro shoot tips. Accordingly, loading might be capable of enhancing the permeation of PVS2 and provide desired degree of desiccation for red bud taro shoot tips. Our results appeared to agree with those of Sarab *et al.* (2012).

In the encapsulation-vitrification method, shoot tips are usually dehydrated by PVS2 (Manar *et al.*, 2012). Therefore, the keys to success for cryopreservation by encapsulation-vitrification are to carefully control the procedure for dehydration with PVS2, which requires the optimum exposure time and temperature to PVS2. To establish this objective, a treatment procedure at about  $25^\circ\text{C}$  or  $0^\circ\text{C}$  has been used (Sant *et al.*, 2008). In the present study, red bud taro shoot tips treated with PVS2 for 50 min at  $0^\circ\text{C}$  or 20 min at  $25^\circ\text{C}$  prior to a direct plunge in LN produced maximum survival rate. Incubation time and temperature in PVS2 appears to be taro-variety-specific and cryopreservation-protocol-specific. In cryopreservation of taro shoot-tips by vitrification, it was 12 min at  $25^\circ\text{C}$  for shoot tips of three cultivars of tropical taro (*C. esculenta* var. *esculenta* (L.) Schott) (Sant *et al.*, 2006) and 20 min at  $25^\circ\text{C}$  for shoot tips of *C. esculenta* var. *antiquorum* cv. ‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ (Takagi *et al.*, 1997). But in the cryopreservation of shoot-tips of *C. esculenta* var. *esculenta* (L.) Schott cultivars by droplet vitrification, exposure time and temperature to PVS2 was 20–40 min at  $0^\circ\text{C}$  instead of  $25^\circ\text{C}$  (Sant *et al.*, 2008), which improved the survival rates to 73%–100% from 21%–30% obtained with vitrification protocol (Sant *et al.*, 2006). But the reason why the dehydration of PVS2 at  $25^\circ\text{C}$  or  $0^\circ\text{C}$  resulted in higher shoot survival are unclear.

The duration of storage generally does not affect the survival of the shoot tips stored in LN (Yin and Hong, 2010). The maximal storage duration is theoretically unlimited, provided that the samples are permanently kept at or near the temperature of liquid nitrogen (Engelmann, 1991). Both Wang *et al.* (2005b) and Tsai *et al.* (2009) have also demon-

strated successful regeneration of shoot-tips of papaya following storage in LN for 2 months and 2 years respectively.

In the current study, the shoot tips of red bud taro have been successfully recovered following liquid nitrogen storage for 150 days with the same high survival rate as the shoot tips cryopreserved for one day. Our results were similar to those obtained in the cryopreservation of *Carica papaya* (Kaity *et al.* 2013).

However, in cryopreservation, rewarming has been considered to be critical (Engelmann, 1991; Guzmán-García *et al.*, 2013; Hülya *et al.*, 2013). In the majority of the cases, rewarming is carried out rapidly by immersing the cryotubes containing the samples in a water-bath thermostated at around 40 °C (Hong and Yin, 2012b). Our results showed rewarming at higher temperature (40 °C) had a much higher survival rate than that at lower temperature (25 °C and 45 °C). Results from this study are consistent with results obtained with other taro varieties. Sant *et al.* (2006) found when shoot-tips of three cultivars of tropical taro (*C. esculenta* var. *esculenta* (L.) Schott) (E399, CPUK and TNS) were warmed by rapid shaking in a water bath at 40 °C for 1 min 30 following cryopreservation by vitrification, the best mean recoveries were 21%, 29% and 30% respectively. Takagi *et al.* (1997) reported that after rapid warming in a water bath at 40 °C following cryopreservation by vitrification, the shoot tip average recovery rate of *C. esculenta* var. *antiquorum* cv. ‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ shoot tips could reach 83.1% and 67% respectively. However, following Cryopreservation by droplet vitrification, the post-thaw survival rates of shoot tips of tropical taro (*C. esculenta* var. *esculenta* (L.) Schott) cultivars reached 73%–100% (Sant *et al.*, 2008).

According to viewpoints of Sant *et al.* (2006; 2008) and Takagi *et al.* (1997), it was essential to plate the shoot tips with the unloading for the re-growth of taro shoot tips. They also noted that unloading using 1.2 mol · L<sup>-1</sup> sucrose was beneficial to post-thaw cultures of *C. esculenta* var. *antiquorum* cv.

‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ shoot tips (Takagi *et al.*, 1997) and unloading using liquid MS with 1.2 mol · L<sup>-1</sup> sucrose was favorable for regeneration of tropical taro (*C. esculenta* var. *esculenta* (L.) Schott) shoot tips (Sant *et al.*, 2006, 2008). In the present study, unloading third with liquid MS medium supplemented with 1.2 mol · L<sup>-1</sup> sucrose also could increase the survival rate of cryopreserved red bud taro shoot tips. Our results also indicated when shoot tips were post-cultured in the dark for 3 days and then transferred to the light conditions, the survival rate was maximum. However, when shoot tips were directly post-cultured under 16 h photoperiod the survival rate decreased significantly. This suggests that culture in the dark immediately after rewarming may contribute to improve recovery by limiting the detrimental photooxidation phenomena (Benson *et al.*, 1989), as has been reported in tropical taro (*C. esculenta* var. *esculenta* (L.) Schott) cultivars maintained in the dark for three days and then transferred to dim light (3.5 μmol · m<sup>-2</sup> s<sup>-1</sup>) for one week (Sant *et al.*, 2006) or cultured overnight in the dark (Sant *et al.*, 2008) and *C. esculenta* var. *antiquorum* cv. ‘Eguimo’ and *C. esculenta* var. *antiquorum* cv. ‘Dodare’ kept in dim light for 10 days prior to exposure under the light conditions (Takagi *et al.*, 1997).

In conclusion, the encapsulation vitrification procedure appears to be a simple and efficient method for cryopreservation of red bud taro shoot tips. Further studies should be directed at examining the applicability of this technique across taro genotypes.

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